OIPE IAP87 APR 19 2007 PATENT & TRADEMARK OFFICE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:			
	Lior GEPSTEIN et al.		
Serial No.:	10/759,734		
Filed:	01/20/2004	Group Art Unit:	1632
For:	METHODS OF GENERATING HUMAN CARDIAC CELLS AND TISSUES AND USES THEREOF		
		Attorney Docket:	27395
Examiner:	SINGH, ANOOP KUMAR		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF MICHAL AMIT UNDER 37 CFR 1.131

I, Michal Amit, am a co-inventor of the above-identified application.

I have read the Office Action mailed on October 19, 2006 with respect to the above-identified application

In the Office Action, the Examiner rejected claims 176-177, 186-189 and 194-195 under 35 U.S.C. 102 (e) over Xu et al (US 2005/0164382A1)

As the Examiner noted in his action that the effective filing date of the Xu reference US 2005/0164382A1 is July 12, 2001, while the publication date of another reference cited by the Examiner, Kehat et al., (*Circulation*, Supplement II, Vol. 102 No. 18, October 31, 2000, abstract IDS, hereinafter "*Circulation*") is October 2000.

The aforementioned *Circulation* publication described the work of mine and my co-inventors, Izhak Kehat, Lior Gepstein, and Joseph Itskovich-Eldor, and discloses our invention. The *Circulation* article describes both the conception and reduction to practice, and thus the making, of the invention disclosed and claimed in the above-identified application, which application was filed on July 20, 2001, well within the one-year grace period permitted by U.S. Patent law.

The Xu et all patent application is therefore not prior art relative to our invention.

APR 19 2007
PATENT & TRADEMARK OFFICE

I hereby declare that all the statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code and the such willful false statements may jeopardize the validity of the application or any patent issued hereon.

Date: April 2, 2007

Amit Michal

Dr Michal Amit

OIPE GIAP97 APR 19 2007 PATENT & TRADEMARK OFFICE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:			
	Lior GEPSTEIN et al.		
Serial No.:	10/759,734		
Filed:	01/20/2004	Group Art Unit:	1632
For:	METHODS OF GENERATING HUMAN CARDIAC CELLS AND TISSUES AND USES THEREOF		
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Examiner:	SINGH, ANOOP KUMAR		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF MICHAL AMIT UNDER 37 CFR 1.132

I, Michal Amit, am a co-inventor of the above-identified application.

I have read the Office Action issued with respect to the above-identified application.

In the Office Action, the Examiner rejected claims 176-181, 186-189 and 194-195 under 35 U.S.C. 102 (a) over Kehat et al (*Circulation*, Supplement II, Vol. 102 NO. 18, October 31, 2000, abstract IDS, hereinafter "*Circulation*")

The Kehat publication is the work of Applicants which was published within one year of the filing date of the instant application and cannot be used against Applicants. *In re Katz*, 687 F.2d 450, 215 USPTO 14 (CCPA 1982).

Dorit Karsenti, Mirit Drukman, and Yair Feld, are identified as co-authors of the above *Circulation* article for their assistance in the development of the invention by carrying out assignments and work under the supervision and direction of myself and my co-inventors, and in their assistance in the preparation of the *Circulation* article, and were therefore not included as co-inventors.

As such, the *Circulation* reference cannot be used as prior art against the instant application since the publication described our own work and was published within one year of our filing date.

I hereby declare that all the statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code and the such willful false statements may jeopardize the validity of the application or any patent issued hereon.

Date: April 2, 2007

Amit Michal

Dr Michal Amit

OIPE IAP97
APR 19 2007
PATENT & TRADEMARK OFFICE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	Lior GEPSTEIN et al.		
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		Attorney Docket:	27395
Examiner:	SINGH, ANOOP KUMAR		

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P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF MICHAL AMIT UNDER 37 CFR 1.132

I am presently employed as a researcher at the Technion Institute of Technology, Haifa Israel, Faculty of Medicine and Rambam Medical Center, Stem Cell Center, where I am a Senior Scientist. I received my Ph.D. degree from the Technion Institute of Technology in Medical Sciences.

My research focuses on derivation and culturing of human embryonic stem cells. Since the beginning of my career, I have published 15 scientific articles in highly regarded journals and books and have presented my achievements at many international scientific conferences.

I am a member of the Internaitonal Society for Stem Cell Research (ISSCR).

I am a co-inventor of the subject-matter claimed in the above-referenced U.S. patent application and a co-author of the Itskovitz-Eldor publication (Mol Med. 2000 Feb;6(2):88-95).

I have read the Official Action issued in the above-identified application.

In this Official Action, the Examiner has rejected claims 176-181, 186-189 and 194-195 under 35 U.S.C. 102(a) as being anticipated by Itskovits-Eldor et al. supra. The Examiner states that Itskovits-Eldor et al. teach an in vitro culture of human cells of cardio specific lineage obtained from human ES cells that shows the cardiac specific synchronous rhythmic activity.

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As a co-author of the Itskovitz-Eldor reference I present the following results which conclusively show that only **one pulsing EB** (i.e., cardiac phenotype) was obtained in the studies presented by the Itskovitz-Eldor reference and therefore cannot anticipate the present invention as now claimed.

Experiments for cardiomyocytes differentiation:

EB formation and differentiation – as described in Itskovitz-Eldor et al. (see page 89 "cell culture" section). The different hES clones used are listed below.

Results

None of the resulting EBs from any of the folowing experiments effected with many cells of different hES clones (number of cells indicated), but the single EB shown in the Itskovitz-Eldor reference, demonstrated a cardiac phenotype (i.e., contractions) indicating the existance of cardiomyocytes.

H13 p29 $2x10^6$ cells

H1 p34 $3x10^6$ cells

H13 p30 $7x10^6$ cells

H1 p36 $3x10^6$ cells

H13 p31 $4x10^6$ cells

H9 p37 $7x10^6$ cells

H9 p37 $10x10^6$ cells

H9 p37 $10x10^6$ cells

H13 p31 $7x10^6$ cells

H1 p37 $18x10^6$ cells

H13 p32 $7x10^6$ cells

H1 p37 $10x10^6$ cells

H13 p32 $6x10^6$ cells

The only one pulsing EB was from H9 p37 which is shown in the Itskovitz-Eldor reference.

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Date: April 19, 2007

Michal Amit

Dr Michal Amit

APR 19 2007
PATENT AND TRADEMARK OFFICE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:		§		
	Lior GEPSTEIN et al.	§		
Serial No.:	10/759,734	§		
Filed:	01/20/2004	§	Group Art Unit:	1632
For:	METHODS OF GENERATING HUMAN CARDIAC CELLS AND TISSUES AND USES THEREOF	§		
		§	Attorney Docket:	**27395**
Examiner:	SINGH, ANOOP KUMAR	§		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF JOSEPH ITSKOVICH-ELDOR UNDER 37 CFR 1.131

I, Joseph Itskovitz-Eldor, am a co-inventor of the above-identified application.

I have read the Office Action mailed on October 19, 2006 with respect to the above-identified application

In the Office Action, the Examiner rejected claims 176-177, 186-189 and 194-195 under 35 U.S.C. 102 (e) over Xu et al (US 2005/0164382A1)

As the Examiner noted in his action that the effective filing date of the Xu reference US 2005/0164382A1 is July 12, 2001, while the publication date of another reference cited by the Examiner, Kehat et al., (*Circulation*, Supplement II, Vol. 102 No. 18, October 31, 2000, abstract IDS, hereinafter "*Circulation*") is October 2000.

The aforementioned *Circulation* publication described the work of mine and my co-inventors, Izhak Kehat, Lior Gepstein, and Michal Amit, and discloses our invention. The *Circulation* article describes both the conception and reduction to practice, and thus the making, of the invention disclosed and claimed in the above-identified application, which application was filed on July 20, 2001, well within the one-year grace period permitted by U.S. Patent law.

The Xu et all patent application is therefore not prior art relative to our invention.

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I hereby declare that all the statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code and the such willful false statements may jeopardize the validity of the application or any patent issued hereon.

Date: April 2, 2007

J. Itskovitz-Eldor

Dr Joseph Itskovitz-Eldor

OIPE APR 19 2007 PATENT & TRADEMARK OFFICE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:			
	Lior GEPSTEIN et al.		
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Commissioner for Patents
P.O. Box 1450
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DECLARATION OF JOSEPH ITSKOVITZ-ELDOR UNDER 37 CFR 1.132

I, Joseph Itskovitz-Eldor, am a co-inventor of the above-identified application.

I have read the Office Action issued with respect to the above-identified application

In the Office Action, the Examiner rejected claims 176-181, 186-189 and 194-195 under 35 U.S.C. 102 (a) over Kehat et al (*Circulation*, Supplement II, Vol. 102 NO. 18, October 31, 2000, abstract IDS, hereinafter "*Circulation*")

The Kehat publication is the work of Applicants which was published within one year of the filing date of the instant application and cannot be used against Applicants. *In re Katz*, 687 F.2d 450, 215 USPTO 14 (CCPA 1982).

Dorit Karsenti, Mirit Drukman, and Yair Feld, are identified as co-authors of the above *Circulation* article for their assistance in the development of the invention by carrying out assignments and work under the supervision and direction of myself and my co-inventors, and in their assistance in the preparation of the *Circulation* article, and were therefore not included as co-inventors.

As such, the *Circulation* reference cannot be used as prior art against the instant application since the publication described our own work and was published within one year of our filing date.

I hereby declare that all the statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code and the such willful false statements may jeopardize the validity of the application or any patent issued hereon.

Date: April 2, 2007

Dr Joseph Itskovitz-Eldor

OIPE IAP97 APR 19 2007 PATENT & TRADEMARK OFFICE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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Examiner:	SINGH, ANOOP KUMAR		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF LIOR GEPSTEIN UNDER 37 CFR 1.131

I, Lior Gepstein, am a co-inventor of the above-identified application.

I have read the Office Action mailed on October 19, 2006 with respect to the above-identified application

In the Office Action, the Examiner rejected claims 176-177, 186-189 and 194-195 under 35 U.S.C. 102 (e) over Xu et al (US 2005/0164382A1)

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The aforementioned *Circulation* publication described the work of mine and my co-inventors, Izhak Kehat, Michal Amit and Joseph Itskovich-Eldor, and discloses our invention. The *Circulation* article describes both the conception and reduction to practice, and thus the making, of the invention disclosed and claimed in the above-identified application, which application was filed on July 20, 2001, well within the one-year grace period permitted by U.S. Patent law.

The Xu et all patent application is therefore not prior art relative to our invention.

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PATENT & TRADEMARK OFFICE

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Date: April 2, 2007

L. Gepstein

Dr Lior Gepstein

OIPE IAP97 APR 19 2007 PATENT & TRADEMARK OFFICE

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Alexandria, VA 22313-1450

DECLARATION OF LIOR GEPSTEIN UNDER 37 CFR 1.132

I, Lior Gepstein, am a co-inventor of the above-identified application.

I have read the Office Action issued with respect to the above-identified application

In the Office Action, the Examiner rejected claims 176-181, 186-189 and 194-195 under 35 U.S.C. 102 (a) over Kehat et al (*Circulation*, Supplement II, Vol. 102 NO: 18, October 31, 2000, abstract IDS, hereinafter "*Circulation*")

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Dorit Karsenti, Mirit Drukman, and Yair Feld, are identified as co-authors of the above *Circulation* article for their assistance in the development of the invention by carrying out assignments and work under the supervision and direction of myself and my co-inventors, and in their assistance in the preparation of the *Circulation* article, and were therefore not included as co-inventors.

As such, the *Circulation* reference cannot be used as prior art against the instant application since the publication described our own work and was published within one year of our filing date.

OIPE IAP97
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Date: April 2, 2007

Dr Lior Gepstein

OIPE IAP97 APR 19 2007 PATENT & TRADEMARK OFFICE

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Examiner:	SINGH, ANOOP KUMAR		

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Alexandria, VA 22313-1450

DECLARATION OF IZHAK KEHAT UNDER 37 CFR 1.131

I, Izhak Kehat, am a co-inventor of the above-identified application.

I have read the Office Action mailed on October 19, 2006 with respect to the above-identified application

In the Office Action, the Examiner rejected claims 176-177, 186-189 and 194-195 under 35 U.S.C. 102 (e) over Xu et al (US 2005/0164382A1)

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Date: April 2, 2007

Dr Kehat Izhak

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Examiner:	SINGH, ANOOP KUMAR		

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Alexandria, VA 22313-1450

DECLARATION OF IZHAK KEHAT UNDER 37 CFR 1.132

I, Kehat Izhak, am a co-inventor of the above-identified application.

I have read the Office Action issued with respect to the above-identified application

In the Office Action, the Examiner rejected claims 176-181, 186-189 and 194-195 under 35 U.S.C. 102 (a) over Kehat et al (*Circulation*, Supplement II, Vol. 102 NO. 18, October 31, 2000, abstract IDS, hereinafter "*Circulation*")

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Date: April 2, 2007

Dr Kehat Izhak

APR 19 2007
PATENT & TRADEMARK OFFICE

Curriculum Vitae - Michal Amit

Personal details:

Name:	Michal Amit
I.D.	023508971
Birth	20.01.1968, Israel
Marital status	Married + 2 children
Home Address:	Yuvalim 261, D.N. Misgav 20142, Israel
Phone:	+972-4-8295445
Mobile phone:	0502-063428
Fax:	+972-4-8295435
E-mail:	mamit@tx.technion.ac.il

Education:

1999-2004 **Ph.D.** degree in Medical Sciences
Rappaport Faculty of Medicine
Technion – Israel Institute of Technology
Haifa 31096, Israel.

Ph.D. thesis: "Human embryonic stem cells: derivation, maintenance and differentiation into hematopoietic progeny."
(under the supervision of Prof. Joseph Itzkovitz-Eldor, Dept. Obstetrics & Gynecology, Technion Institute of Technology & Rambam Medical Center, Haifa; Prof. Dov Zipori Weizmann Institute of Science, Rehovot)

1993-1997 **M.Sc.** degree in Medical Sciences
Rappaport Faculty of Medicine
Technion – Israel Institute of Technology
Haifa 31096, Israel.

M.Sc. thesis: "Neurotransmitters in meiotic resumption of mammalian oocytes."
(under the supervision of Prof. Joseph Itskovitz-Eldor, Dept. Obstetrics &

Gynecology, Technion - Israel Institute of Technology & Rambam Medical Center, Haifa)

1990-1993 **B.A.** in Biology
Faculty of Biology
Technion – Israel Institute of Technology
Haifa Israel.

Professional Experience:

1997- 2004 Teaching a course on "Topics in Biology"
Ort Brauda College of Technology, Karmiel, Israel.

2004- Teaching a course on "Tissue culture of animal cells"
Ort Brauda College of Technology, Karmiel, Israel.

2004- 2005 Teaching one day seminar for high school students on "Tissue culture"
Ort Brauda College of Technology, Karmiel, Israel.

2004- Chief instructor of five day course on "Human embryonic stem cells: culture techniques" under the auspices and sponsorship of the National Institutes of Health, USA. The course is conducted twice a year in Johns Hopkins University, Baltimore, Maryland, USA and on an individual basis at the Technion - Israel Institute of Technology.

2005-2005 Teaching a course on "Biology of the cell"
Ort Brauda College of Technology, Karmiel, Israel.

2005- Chief instructor of five day course on "Human embryonic stem cells: culture and analysis techniques" The course is conducted twice a year in Coriell Institute, Camden, Mew Jersey, USA.

Expertise:

1. Derivation, differentiation and maintenance of primate embryonic stem cells. Five months advanced studies under the direction of Prof. J. Thomson at the University of Wisconsin, WI, USA (September 1998 – February 1999).

Research presentations at National and International Meetings:

1. Bar-Ami S., **Amit M.**, Nahir M., and Itskovitz-Eldor J. " γ–Aminobutyric Acid (GABA) induces *in vitro* expansion and hyaluronic acid accumulation in the rat cumulus-oocyte complex". Society for the Study of Reproduction, 30th Annual Meeting, August 1997, Oregon, USA. Vol 56: Abstract 385 pp.179. **Lecture.**

2. **Amit M.**, Margulets V, Segev H, Shariki C, Laevsky I, Coleman R, Itskovitz-Eldor J. "Human feeder layers for human embryonic stem cells". Society for Gynecologic Investigation, 50th Annual Meeting, March 2003, Washington DC, Vol 10 (2); Abstract 746 pp 338a. **Poster.**

3. **Amit M**, Margulets V, Segev H, Shariki K, and Itskovitz-Eldor J. "Alternative culture conditions for human embryonic stem cells". Keystone Symposia "From Stem cells to Therapy" , March-April 2003, Steamboat Spring, Colorado, USA. Abstract 2003 pp 88. **Poster.**

4. **Amit M**, Margulets V, Segev H, Shariki K, Laevsky I, Coleman R, and Itskovitz-Eldor J. "Alternative culture conditions for human embryonic stem cells". The Israeli Society for Infertility Research, Annual Meeting. May 2003, Tel Aviv, Abstract 16. **Lecture.**

5. Suss-Toby E, Gerecht Nir S, **Amit M**, Manor D, and Itskovitz-Eldor J. "Derivation of diploid human pluripotent embryonic stem cell line from mononuclear zygote". The Israeli Society for Infertility Research, Annual Meeting. May 2003, Tel Aviv, Abstract 86. **Poster.**

6. **Amit M**, Margulets V, Shariki K, Segev H, and Itskovitz-Eldor J. "Animal-free culture system for human embryonic stem cells". International Society for Stem Cell Research, Annual Meeting.. June 8-11, 2003, Washington, DC, USA. **Poster** 38.

7. **Amit M**, Margulets V, Shariki K, and Itskovitz-Eldor J. Alternative methods for culturing human embryonic stem cells. NIH Research Meeting: "Recent progress and future promise of human embryonic stem cells". June 12, 2003, Washington, DC, USA. **Poster** 1.

8. **Amit M**, Menke S, Brüning E, Itskovitz-Eldor J, Benvenisty N, Denner J, Winkler M, Martin U. "No evidence for infection of human embryonic stem cells by feeder cell-derived murine leukemia viruses (MLV)". Keystone Symposia " Stem cells", January 2004, Steamboat Spring, Colorado, USA. **Poster 106.**

9. **M Amit**, V Margulets, K Shariki, H Segev, D Manor, and J Itskovitz-Eldor. "Derivation of human embryonic stem cells harboring genetic defects". Keystone Symposia " Stem cells", January 2004, Steamboat Spring, Colorado, USA. **Poster 230**.

10. **M Amit**, K Shariki,V Margulets, and J Itskovitz-Eldor. "Serum and feeder free culture system for human embryonic stem cells". Keystone Symposia " Stem cells", January 2004, Steamboat Spring, Colorado, USA. **Poster 316**.

11. Ulrich M, **Amit M**, Menke S, Bruning E, Winkler M, HaverichA, Itskovitz-Eldor. "No evidence for infection of human embryonic stem cells by feeder cell-derived murine leukemia viruses (MLV)". 2nd International Meeting, Stem Cell Network, North Rhine Westphalia, Germany. **Poster 059**.

12. **M Amit**, V Margulets, K Shariki, and J Itskovitz-Eldor. "Derivation of human embryonic stem cells harboring genetic defects". International Society for Stem Cell Research, 2 th Annual Meeting.. June 10-13, 2004, Boston, USA. **Poster** 38.

13. Kristin Schwanke, Monica Winkler, Andreas Schmiedl, Konstantin Miller, **Michal Amit**, Joseph Itskovitz-Eldor, Axel Haverich, Ulrich Martin. Molecular, ultrastructural and immunohistological characterisation of the Rhesus monkey derived embryonic stem cell line 366.4. Keystone Symposia " Stem cells", February 2005, Steamboat Spring, Colorado, USA. **Poster**.

Invited lectures:

1. "Human embryonic stem cells derivation and spontaneous differentiation." In the course: "News in medical diagnostics" Rambam Medical Center, 6 November 2001.

2. "Human embryonic stem cells: differentiation and clinical uses." Israel Society for the Biology of Aging, Annual Conference, Weizmann Institute of Science, Rehovot, 29 May 2002.

3. "Derivation and differentiation of human embryonic stem cells." Israel Society for Histochemistry and Cytochemistry, Annual Conference, Bar-Ilan University, Ramat Gan, 3 June 2002.

4. Technion Center of Embryonic Stem Cells - Research Progress Report. NIH human embryonic stem cells infrastructure meeting, Washington, D.C., USA June 13, 2003.

5. "Alternative methods for the derivation and culture of human embryonic stem cells." European Society of Human Reproduction and Embryology, Annual Meeting pre-course. Madrid, Spain. June 29, 2003.

6. "Human embryonic stem cells." Israel seminar for high school teachers. Ashdod, 23 March 2004.

7. "Methods for the derivation and culture of human embryonic stem cells." Annual Meeting of the Israel Society for Histochemistry and Cytochemistry. Haifa, 1 June 2004.

8. "From human embryonic stem cells to cloning human beings." Major lecture at the Graduation Ceremony. Ort Brauda College of Technology, Karmiel, Israel, 20.5.04.

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1. 2002-2006 **Ministry of Science, Culture & Sport**, Israel (Itskovitz-Eldor, PI) (Leading Scientist) (Differentiation of human embryonic stem cells into hematopoietic stem cells).

2. 2003-2005 **National Institutes of Health**, USA (Itskovitz-Eldor, PI) (Chief instructor) (Short term course in human embryonic stem cell culture techniques) (with Rao M, collaborator).

3. 2003-2005 **National Institutes of Health**, USA (Itskovitz-Eldor, PI) (leading Scientist) (Infrastructure for stem cells production and distribution).

4. 2004-2008 **Ministry of Industry and Trade**, Israel (Itskovitz-Eldor, PI) (Leading Scientist) (Magnet, Production and scale-up of human embryonic stem cells).

5. 2005-2008 **National Institutes of Health**, USA (Itskovitz-Eldor, PI) (leading Scientist) (Infrastructure for stem cells production and distribution).

6. 2006-2009 **National Institutes of Health**, USA (Itskovitz-Eldor, PI) (Chief instructor) (Short term course in human embryonic stem cell culture techniques) (with Rao M, collaborator).

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Sizing up the heart: development redux in disease

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REVIEW

Sizing up the heart: development redux in disease

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Following gastrulation and establishment of the three embryonic germ layers, the first definitive organ to form in the embryo is the heart, whose morphogenesis, growth, and integrated function are essential to embryonic survival, even by midgestation. Abnormalities in heart development result in congenital heart disease, the most frequent form of birth defects in humans. At the opposite end of the temporal spectrum, adult cardiac disease is the most common cause of death in the industrialized world, with congestive heart failure and inadequate pump function the end result of diverse disorders intrinsic to cardiac muscle cells, cardiac valves, systemic blood pressure, and the coronary blood supply. Despite recent therapeutic advances and mechanical devices to sustain cardiac function, only a minority of heart failure patients lives longer than 5 yr. Death from heart disease therefore comprises an epidemic more prevalent than all cancers combined (Ries et al. 2003).

Recent studies have begun to reveal the cellular circuitry that controls cardiac growth during development and disease. Intriguingly, many of the molecules and mechanisms that regulate growth of the embryonic heart are redeployed in the adult heart in response to stress signals that provoke cardiac enlargement and heart failure. Thus, understanding the mechanisms involved in heart development promises to provide insights into the molecular basis for pathogenesis of the adult heart, as well as to reveal novel therapeutic targets. In this review, we consider three aspects of cardiac development with significant implications for adult heart disease: (1) normal growth during organogenesis, (2) a "fetal" cardiac gene program reactivated in hypertrophy, and (3) restorative growth by undifferentiated progenitor cells that have cardiogenic potential. Each of these aspects of cardiac growth could be, itself, the subject of an in-depth review. Our goal, however, is not to comprehensively review these areas, but to identify common themes in developmental biology that are reiterated in settings of abnormal growth and dysfunction of the adult heart. Although we focus on development and disease of the myocardium, many of the same principles of allometric growth and homeostasis apply to other organs whose structure and function are influenced by physiological and pathological signaling.

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Developmental growth of the heart

Specification of cardiac cell fate by extracellular cues

Heart formation is initiated in vertebrate embryos soon after gastrulation when a population of cells in a region of anterior mesoderm known as the cardiac crescent (Fig. 1) adopts a cardiac fate in response to cues from adjacent endoderm (Olson 2001). Three families of peptide growth factors have been studied most intensely for their positive (and negative) effects on the establishment of cardiac cell identity. Bone morphogenetic proteins (BMPs), a subset of the transforming growth factor-β (TGFβ) superfamily (Zaffran and Frasch 2002; Schneider et al. 2003), promote cardiogenesis in vertebrate embryos (Fig. 2). Similarly, the *Drosophila* ortholog, decapentaplegic (Dpp), its receptors, and effector Smad transcription factors are essential for the formation of the dorsal vessel, the rudimentary heart-like organ in fruit flies. Equivalent effects have been shown in *Xenopus* and avian embryos or explants, using soluble inhibitors of the BMP ligand–receptor interaction such as noggin (Schultheiss et al. 1997), dominant-negative BMP receptors (Shi et al. 2000), or inhibitory Smads (Krishnan et al. 2001; Walters et al. 2001). BMPs also are required to generate cardiac muscle cells from mouse teratocarcinoma cells in culture (Monzen et al. 1999). In mice, mutations of certain BMPs or of any BMP receptor are lethal at gastrulation, obscuring the potential role of BMPs in cardiogenic specification, even though their later role in cardiac morphogenesis was made clear by a heart-specific deletion (Gaussin et al. 2002; Schneider et al. 2003). Notably, epiblast-specific deletion of the type IA BMP receptor is permissive for gastrulation but markedly impairs the inception of heart development (Miura et al. 2002).

Limited studies, chiefly in avians and zebrafish, suggest an analogous cardioinductive role for fibroblast growth factors (Lough et al. 1996; Barron et al. 2000;

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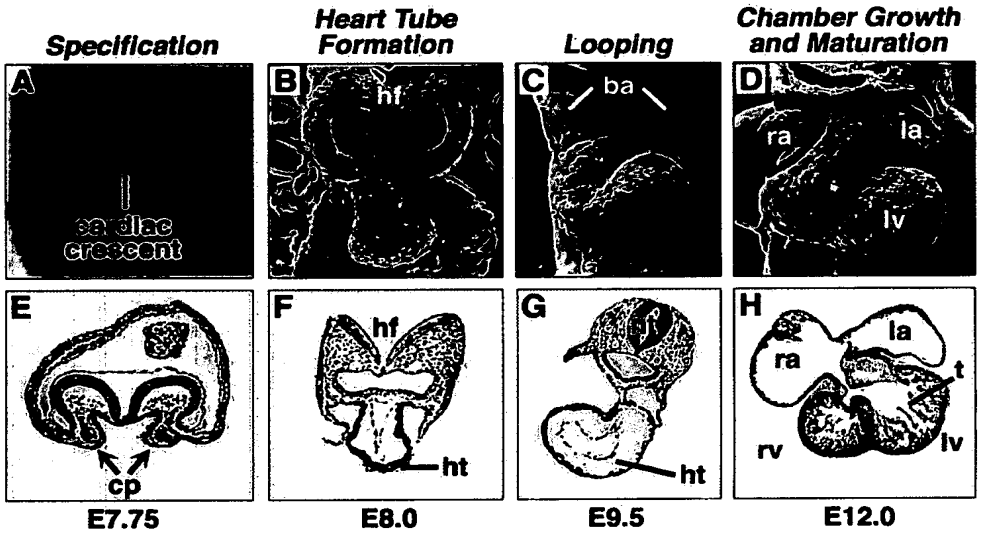

Figure 1. Morphogenesis and growth of the heart. Stages in development of the mouse heart are shown. (*A*) Expression in the cardiac crescent of a lacZ transgene linked to *HAND2* regulatory sequences (McFadden et al. 2000). (*E*) A histological section. (*B–D*) Scanning electron micrographs of the developing heart. (*F,G*) Histological sections of embryos harboring lacZ transgenes linked to the *HAND2* and *Nkx2.5* (Lien et al. 1999) regulatory sequences, respectively, at the indicated stages. The heart is indicated in blue. (ba) Branchial arch; (cp) cardiac progenitors; (hf) head folds; (ht) heart tube; (la) left atrium; (lv) left ventricle; (nt) neural tube; (ra) right atrium; (rv) right ventricle; (t) trabeculae. (Panels *B–D* are reprinted from Kaufman 1992 with permission from Elsevier.)

Reifers et al. 2000; Alsan and Schultheiss 2002). In contrast, Heartless—the *Drosophila* fibroblast growth factor receptor—is required not for specification per se, but rather for cardiac precursor cells to assume their right position to receive the specifying cues (Gisselbrecht et al. 1996).

Wingless in *Drosophila* and the related Wnt proteins in vertebrates comprise the third class of secreted signal for cardiac specification. In this case, however, both induction and suppression have been reported. Wingless is essential for cardiogenesis in flies, as cells exposed just to Dpp become visceral muscle instead (Zaffran and Frasch 2002). Surprisingly, given these results, Wnt signaling blocks cardiogenesis in heart field explants from the chick and in microinjected *Xenopus* embryos overexpressing Wnt3A or Wnt8 (Fig. 2; Marvin et al. 2001; Schneider and Mercola 2001; Tzahor and Lassar 2001). Likewise, inhibitors of the Wnt signaling cascade can induce cardiogenic differentiation ectopically, in presumptive blood-forming cells and anterior paraxial mesoderm. Furthermore, endogenous Wnt antagonists (Crescent, Dickkopf-1) appear to be critical signals from the organizer or anterior endoderm to disinhibit cardiac specification in the adjacent mesoderm.

Making matters more complex is the recent report that Wnt11 stimulates cardiogenesis in avian explants, a quail mesodermal cell line, *Xenopus*, and mouse P19 cells (Eisenberg et al. 1997; Eisenberg and Eisenberg 1999; Pandur et al. 2002). At least in the latter two systems, such procardiogenic signals are transduced through the "noncanonical" Wnt pathway, involving protein kinase C and Jun N-terminal kinase (Pandur et al. 2002). Thus, with regard to cardiogenesis, both positive- and negative-acting Wnts exist.

The primary mode of Wingless/Wnt signaling is through an intracellular cascade that inhibits activity of glycogen synthase kinase-3 (GSK3), which phosphorylates armadillo/β-catenin, a transcriptional coactivator that associates with high-mobility group (HMG)-box transcription factors of the T-cell factor (TCF) family, converting them from repressors to activators (for review, see Harwood 2001). Phosphorylation of β-catenin by GSK3 targets it for degradation by the ubiquitin pathway, thereby suppressing TCF-dependent transcription. Consistent with a suppressive effect of Wnts on cardiogenesis, expression in anterior mesodermal cells of activated GSK3, which inhibits signaling from β-catenin to TCFs, can promote cardiogenesis (Schneider and Mercola 2001). The target genes of TCFs that suppress cardiogenesis remain to be identified. Remarkably, conditional deletion of β-catenin in definitive endoderm of the mouse leads to the formation of ectopic hearts (Lickert et al. 2002). Although this experiment was construed as consistent with Wnts' inhibitory effects on heart formation in other vertebrates, ectopic expression of BMP2 resulting from loss of β-catenin is a likely instigating factor.

A Wnt- and β-catenin-dependent pathway for mammalian cardiac myogenesis, resembling the primordial Wnt pathway in flies, is operative and essential for cardiac differentiation in pluripotent mouse P19CL6 cells (Nakamura et al. 2003). In this clonal line, expression of

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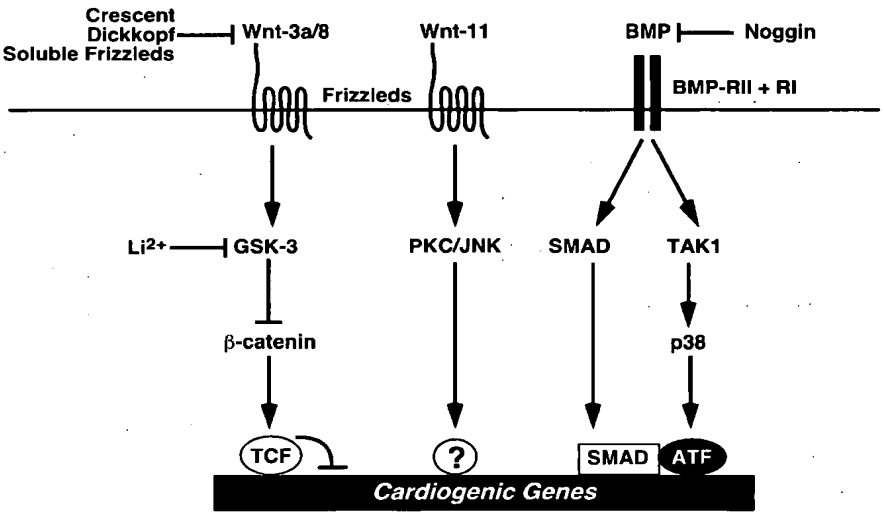

Figure 2. Signaling pathways leading to cardiac specification. Signaling pathways from the membrane to the nucleus involved in mammalian cardiogenesis are shown. Wnts act through seven-transmembrane frizzled receptors. Wnt-3a and Wnt8 act through a canonical signaling pathway involving GSK3 and β-catenin to target TCF, which represses cardiogenesis. The anticardiogenic activity of Wnt-3a/8 can be suppressed by Crescent, Dickkopf and soluble frizzleds in the extracellular milieu, and by Li^{2+}, which inhibits GSK3 activity. Wnt-11, which acts through PKC/JNK, possesses procardiogenic activity; the nuclear target in this pathway is unknown. BMPs act through two receptors, RI and RII, and activate bifurcating signaling cascades mediated by Smad and the kinases TAK and p38, which target ATF transcription factors. The soluble BMP antagonist Noggin suppresses the procardiogenic function of BMP.

BMPs was a relatively late response to the differentiating signal dimethylsulfoxide, Wnt3A and 8A were early transient responses, exogenous Wnt3A and 8A promoted cardiac differentiation, and Wnt or β-catenin inhibitors blocked differentiation. As a potential point of reconciliation with the endoderm-restricted loss of β-catenin, Wnt inhibitors blocked the induction of endogenous BMP2 and BMP4 (Nakamura et al. 2003). Hence, Wnts govern BMP expression in both settings. However, in the absence of molecular markers for the molecularly unspecified cardiac progenitor, a caveat in this clonal system [and also in mouse embryonic stem (ES) cells or pluripotent cells of other kinds] is that the Wnt/β-catenin cascade could act extrinsic to the future cardiomyocyte by induction, expansion, or augmentation of BMP-producing endoderm-like cells. A similar cautionary note pertains to experiments in embryos or explants.

In summary, recent advances reinforce the central, indispensable role of BMP signaling in cardiac myogenesis and later cardiac morphogenesis. The role of Wnts in vertebrate heart formation, first thought to be merely suppressive, is more complex than envisioned, encompassing induction of cardiogenic differentiation by alternative Wnt isoforms, alternative Wnt mediators, and even a Wnt–β-catenin–TCF cassette like the archetypal pathway in flies.

Other extracellular cues may also contribute to cardiac specification, including TGFβ family members beyond just BMPs. Nodal, for instance, has been implicated in heart formation, in large part from the reduction in myocardium in zebrafish mutants (*one-eyed pinhead*) lacking its coreceptor, a member of the epidermal growth factor (EGF) family (Griffin and Kimelman 2002). An analogous role is reported, in mice, for Cripto, the founding member of this family (Xu et al. 1998, 1999). However, a complication akin to that detailed earlier for the exact role played by Wnts is that mesoderm and endoderm are defective, much more generally, in both mice and zebrafish lacking these signaling molecules (Ding et al. 1998; Gritsman et al. 1999).

Specification of cardiac cell fate by multiprotein complexes of cardiogenic transcription factors

Anterior mesodermal cells receiving the appropriate set of signaling inputs switch on a set of cardiac-restricted transcription factors that interact in combinatorial ways to control downstream genes in the cardiac pathway. The homeodomain transcription factor Nkx2.5 (Lints et al. 1993) and the T-box protein Tbx5 (Bruneau et al. 1999; Horb and Thomsen 1999) are among the earliest markers of the cardiac lineage. Forced expression of Nkx2.5 in *Xenopus* and zebrafish embryos can expand the heart-forming region (Chen and Fishman 1996; Cleaver et al. 1996), whereas expression of dominant negative-Nkx2.5 (Tonissen et al. 1994; Evans et al. 1995) and Tbx5 mutant proteins eliminates the heart (Horb and Thomsen 1999). Nkx2.5 and Tbx-5 associate with members of the GATA family of zinc-finger transcription factors and with serum response factor (SRF), a MADS (MCM1, Agamous, Deficiens, serum response factor) box transcription factor, to activate cardiac structural genes (Chen and Schwartz 1996; Sepulveda et al. 1998, 2002; Belaguli et al. 2000; Bruneau et al. 2001). The *Nkx2.5* and *GATA* genes are also regulated by Nkx2.5 and GATA factors, fulfilling positive feedback loops that amplify and maintain their expression following entry of cells into the cardiac lineage (Schwartz and Olson 1999; Molkentin et al. 2000). Ectopic expression of GATA5 can also induce cardiac genes and beating foci of myocardial tissue in zebrafish embryos, whereas heart formation is compromised in fish lacking GATA5 (Reiter et al. 1999). However, none of these factors alone or in combinations can induce endogenous cardiac gene expression in cul-

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tured cells, suggesting that other factors and/or signals that coexist in vivo are required for their ability to confer cardiac induction. Alternatively, the cardiac program might be responsive only to a very precise stoichiometry of these factors that is difficult to achieve in ectopic expression assays.

Despite their apparent importance in early cardiac development, deletion of the genes encoding Nkx2.5, Tbx5, and GATA factors in knockout mice does not prevent specification or differentiation of cardiomyocytes, but instead results in later abnormalities in cardiac morphogenesis (Lyons et al. 1995; Kuo et al. 1997; Molkentin et al. 1997; Tanaka et al. 1999; Bruneau et al. 2001). The lack of early cardiac phenotypes in these mutants is likely to reflect redundant mechanisms for regulation of the initial steps in the cardiac pathway, including structural homologs in each of these three families as well as functional redundancy by unrelated proteins.

A critical role for SRF in cardiogenesis has been inferred from transfection assays demonstrating the essential role of SRF binding sites for cardiac gene transcription (Chen and Schwartz 1996; Reecy et al. 1999) and from the especially high levels of SRF in the developing heart (Croissant et al. 1996). However, the function of SRF in heart development in vivo has been clouded by the early lethality of SRF null mice prior to the onset of cardiogenesis (Arsenian et al. 1998). SRF recruits a powerful transcriptional coactivator, called myocardin, to activate transcription of cardiac genes (Wang et al. 2001). A myocardin dominant-negative mutant protein can prevent heart formation in *Xenopus* embryos (Wang et al. 2001), and forced expression of myocardin can activate ectopic cardiac gene expression even in spinal cord neurons of transgenic frogs (E. Small, E. Olson, and P. Krieg, unpubl.). Myocardin also acts as a master regulator of smooth muscle genes, suggesting commonalities in the cardiac and smooth muscle gene programs (J. Chen et al. 2002; Du et al. 2003; Wang et al. 2003; Yoshida et al. 2003). It is interesting to note, in this regard, that the early heart tube resembles a primitive vessel and transiently expresses smooth muscle genes. Cysteine-rich LIM-only proteins serve as especially potent coactivators of SRF and GATA factors in the context of smooth muscle differentiation, even activating the endogenous smooth muscle gene program (Chang et al. 2003). Given the right combination of SRF, bridging factors, and tissue-specific cofactors, a related complex might be expected to be instrumental in cardiogenesis as well.

Control of cardiomyocyte differentiation

Unlike skeletal muscle cells, in which proliferation and differentiation are mutually exclusive, embryonic cardiac myocytes differentiate and assemble functional sarcomeres even while they proliferate, although the extent of sarcomere organization is far less than after birth. Members of the myocyte enhancer factor-2 (MEF2) family of transcription factors play a key role in the control of cardiomyocyte differentiation by switching on cardiac muscle structural genes whose products participate in myofibrillogenesis and muscle metabolism (for review, see McKinsey et al. 2002). The four vertebrate MEF2 genes (*MEF2A*, *MEF2B*, *MEF2C*, and *MEF2D*) are expressed in overlapping patterns throughout the developing heart. Mice lacking MEF2C die at the onset of cardiac looping and show severe ventricular hypoplasia with reduced expression of a subset of cardiac structural genes (Lin et al. 1997). Likewise, the single *MEF2* gene in *Drosophila* is required for differentiation of cardiac muscle cells and dispensable for initial steps in cardiac lineage commitment and heart tube patterning (Bour et al. 1995; Lilly et al. 1995; Ranganayakulu et al. 1995). A very different phenotype results from deletion of MEF2A—a deficiency of cardiac mitochondria and susceptibility to sudden death, which is attributed to the very different developmental regulation of MEF2A, the predominant MEF2 protein in postnatal hearts (Naya et al. 2002). It is unknown whether differences in MEF2 target-site specificity or MEF2-binding proteins also contribute to functional differences among the four family members. Given that MEF2 in adult hearts provides a nexus for repression of certain "fetal" cardiac genes in the absence of hypertrophic signals (a point discussed at length following), it is intriguing to speculate how MEF2-dependent transcription becomes permissible during embryogenesis; if analogy to skeletal muscle holds true, equivalent processes might be involved at both stages.

Morphogenesis and growth of the embryonic heart

Soon after their specification, cardiac precursor cells from the cardiac crescent converge along the ventral midline of the embryo to form the linear heart tube, which undergoes looping, chamber growth and specification, and septation to ultimately generate the structure of the multichambered heart (Fig. 1; for reviews, see Fishman and Chien 1997; Srivastava and Olson 2000; Harvey 2002). Looping morphogenesis, the earliest of left–right asymmetries in the developing organism, is regulated by a complex network of growth factors, but even may involve patterning, in *Xenopus*, at the four-cell stage (Levin et al. 2002). Growth of the primary heart tube has been shown to occur through ballooning of the outer curvature of the ventricular segments (Christoffels et al. 2000). In addition to the many genes discussed following, intracardiac flow forces play a key epigenetic role in fashioning the early heart (Hove et al. 2003). Because our focus here is specifically on those trophic and transcriptional circuits with most immediate relevance for mechanisms or countermeasures in adult disease, we direct readers elsewhere for coverage of other topics (Rosenthal and Xavier-Neto 2000; Srivastava 2001; Tabin and Vogan 2003).

The phenotypes of mouse and zebrafish mutants have led to the notion that the heart develops in a segmental fashion, with the growth of each chamber dependent on distinct combinations of transcription factors (Fishman and Olson 1997). This is consistent with the abnormalities associated with hypoplastic heart syndromes in humans, in which one ventricular chamber or the other is

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underdeveloped or absent and the other is largely normal. During mouse embryogenesis, the basic helix–loop–helix transcription factors, HAND1 and HAND2, are expressed in highly restricted patterns in the developing left and right ventricular chambers, respectively (Srivastava et al. 1995). In mice lacking HAND2, the right ventricular chamber fails to grow; instead, presumptive right ventricular myocytes undergo apoptosis resulting in a lethal hypoplastic ventricular phenotype (Srivastava et al. 1997; Thomas et al. 1998). This cardiac phenotype is reminiscent of that of embryos lacking the SET (position effect variegation suppressor Su(Var)3-9, Enhancer of Zeste, Trithorax)-domain protein BOP, a cardiac-restricted transcriptional repressor that is regulated by HAND2 (Gottleib et al. 2002). Placental abnormalities in HAND1 mutant mice have complicated the analysis of the role of this gene in cardiac development (Firulli et al. 1998; Riley et al. 1998), although HAND1 null ES cells fail to contribute to the left ventricle in chimeric mice (Riley et al. 2000). Cardiac-specific deletion of HAND1 results in severe cardiac malformations as well (D. McFadden, A. Barbosa, and E.N. Olson, unpubl.). The "floxed" allele also provides an opportunity to test the role of HAND1 in adult myocardium, especially as HAND1 is down-regulated during cardiomyopathy (Natarajan et al. 2001; Thattaliyath et al. 2002). Further evidence for an obligate role of HAND genes in ventricular growth comes from the analysis of mice lacking Nkx2.5, which fail to express HAND1 in the future left ventricular region and die at midgestation from a block in cardiac morphogenesis (Biben and Harvey 1997). In mice lacking HAND2 and Nkx2.5, neither HAND gene is expressed in the heart, resulting in a near complete absence of ventricular myocardium (Yamagishi et al. 2001). However, this phenotype is much more severe than that of mice with a cardiac-specific deletion of HAND1 combined with a HAND2 deletion (D. McFadden, A. Barbosa, and E. Olson, unpubl.), suggesting that Nkx2.5 has essential downstream target genes in addition to HAND1.

Tbx5 is also required for proper growth of the myocardium. Mutations in Tbx5 are responsible for Holt-Oram syndrome, which is characterized by a spectrum of cardiac defects including hypoplastic left ventricle, atrial–septal defects, and ventricular–septal defects (Bruneau et al. 2001). As in the majority of other cardiac mutants, the target genes of this transcription factor responsible for these structural abnormalities remain to be defined.

There is an intriguing linkage between abnormalities in growth and patterning of the heart and limbs such that human syndromes, such as Holt-Oram syndrome, are associated with malformations in both structures. In Holt-Oram syndrome, missense mutations in different domains of Tbx5 preferentially cause heart or hand abnormalities, which supports the notion that Tbx5 may engage different cofactors to govern the growth and patterning of these tissues (Basson et al. 1999). HAND2 has also been implicated in limb patterning. In mice and zebrafish lacking HAND2, limb bud outgrowth is impaired, reflecting the failure of the morphogen sonic hedgehog to be expressed in the zone of polarizing activity (Charite et al. 2000; Yelon et al. 2000). Conversely, misexpression of HAND2 in the anterior compartment of the limb bud is sufficient to generate an ectopic zone of polarizing activity with resulting polydactyly and posterior digit duplication. As mentioned subsequently, other lessons from the limb bud to the heart may include a better understanding of mechanisms underlying successful regenerative growth.

Extracellular signals for ventricular growth

The identification of essential mitogens for cardiomyocyte growth during embryogenesis may have pivotal implications of two kinds after birth: for pathways that confer just cell enlargement once irreversible cell cycle exit has transpired, and for the proliferation of potential regenerative cells. Prior to embryonic day 9.5 (E9.5) in the mouse, the looped heart tube is a thin-walled structure with the atrial and ventricular chambers molecularly specified, but indistinct (Fig. 1). Growth of the heart from this stage onward involves proliferation of myocytes along the walls of the heart tube and within the developing interventricular septum. The most highly proliferative cardiomyocytes are located along the outer surface of the heart, a region called the compact zone (Fig. 1). Fate mapping experiments have shown that individual cardiomyocyte precursors give rise to cone-shaped growth units within the ventricular wall (Mikawa et al. 1992). As the wall thickens, cardiomyocytes along the inner wall become organized into fingerlike projections, called trabeculae, which are thought to enhance oxygen and nutrient exchange and force generation.

The epicardium, the thin layer of cells surrounding the heart, serves as a source of mitogenic signals that are necessary and sufficient to stimulate proliferation of cardiomyocytes within the compact zone. Retinoic acid (RA) produced by the epicardium is one critical regulator of cardiac growth. Knockout mice lacking the RA receptor RXRα die during embryogenesis from a failure in proliferative expansion of ventricular cardiomyocytes resulting in a thin-walled ventricle (Sucov et al. 1994; Kastner et al. 1997). This cardiac defect is recapitulated by epicardium-specific deletion of RXRα, but not by cardiomyocyte-specific deletion of the gene, confirming that the effects of RA on cardiac growth are nonmyocyte autonomous, as concluded also from chimeric embryos (Chen et al. 1998; Tran and Sucov 1998). Consistent with the role of the epicardium as a source of RA for myocardial growth, the epicardium expresses high levels of retinaldehyde-oxidizing dehydrogenase (RALDH2), a key RA synthetic enzyme, and an RA-sensitive lacZ reporter gene is highly expressed in the epicardium and developing heart (Moss et al. 1998). In addition to these effects emphasizing the contribution of RA to ventricular growth, other functions of this pathway are noteworthy, such as the requirement for RA in posterior chamber specification, shown by the knockout of the *RALDH2* gene (Niederreither et al. 2001) and by administration of ectopic RA (Xavier-Nato et al. 1999).

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Mice lacking erythropoietin (epo) or the epo receptor show cardiac growth defects ascribed to another non-myocyte autonomous mechanism (Wu et al. 1999) involving the ability of RA and epo to promote cardiomyocyte proliferation in the presence of epicardium, but not in its absence (T.H. Chen et al. 2002; Stuckmann et al. 2003). The identity of the epicardium-dependent cardiac mitogen remains to be determined.

Growth signals from the endocardium, the specialized endothelial lining of the heart, are critical as well. The neuregulin family of peptide growth factors and their tyrosine kinase receptors (ErbBs), first implicated in oncogenic signaling in breast cancer cells, have also been shown to promote growth of embryonic cardiomyocytes in vivo (Zhao et al. 1998) and play multiple roles in the intact heart (for review, see Garratt et al. 2003). Knockout mice lacking ErbB2 (a coreceptor for other Erbs), Erb4, or neuregulin-1 die at midgestation from cardiac growth defects characterized by the absence of trabeculae (Gassmann et al. 1995; Lee et al. 1995). This abnormality can be ascribed to the lack of paracrine signaling between the endocardium and myocardium. Mice lacking the ShcA adaptor protein, which signals downstream of ErbB receptors, show a similar cardiac growth phenotype (Lai and Pawson 2000). Cardiac-specific deletion of ErbB2 results in dilated cardiomyopathy with ventricular wall thinning in adult animals (Crone et al. 2002) reminiscent of the cardiac phenotype observed in some breast cancer patients receiving anti-ErbB2 antibody therapy. The precise etiology of this form of cardiomyopathy remains to be determined, but may involve the perturbation of a cardiac cell survival pathway. Neuregulins have also gained interest as an inductive signal for the heart's specialized conduction system (Rentschler et al. 2002).

Nuclear regulators of cardiac growth

Several genes with key roles in the control of cardiac growth during embryogenesis have recently been discovered. One such gene encodes a homeodomain called homeodomain-only protein (HOP) that appears to play a dual role in the control of cardiac growth during embryogenesis and early prenatal development (F. Chen et al. 2002; Shin et al. 2002). During mid-embryogenesis, HOP is expressed predominantly in the trabecular region of the myocardium (where cardiomyocyte proliferation is diminished). HOP null mice segregate into two classes that exhibit either an excess or deficiency of cardiac myocytes. Mutants with myocyte deficiency die during early embryogenesis as a result of cardiac insufficiency. Paradoxically, embryos that can survive this critical period show an excess of cardiac myocytes after birth. These antithetical phenotypes suggest a role for HOP in regulating the balance between proliferation and differentiation, which may depend on the spectrum of available partners for HOP as well as the signals received by the cardiac myocyte at a given time and place in the developing heart. Alternatively, the increased number of cardiomyocytes in survivors may reflect a successful secondary adaptation.

Genetic screens in zebrafish have also led to the identification of novel genes that affect cardiac growth. Among these is Reptin, a component of a DNA-stimulated ATPase complex that is associated with Pontin, thought to be a DNA helicase that antagonizes Reptin activity (Rottbauer et al. 2002). Reptin and Pontin bind the β-catenin/TCF complex, a critical mediator of Wnt signaling discussed earlier. Consistent with their proposed antagonistic roles, Reptin is a transcriptional corepressor for β-catenin/TCF-dependent repression, whereas Pontin acts in an opposing manner. In zebrafish embryos bearing an activating mutation of Reptin, cardiomyocytes exhibit a cell-autonomous excess in proliferation. Reducing the expression of Pontin phenocopies the hyperplasic effect of Reptin, supporting the notion that Reptin and Pontin act in antagonistic manners to drive and suppress cardiomyocyte proliferation, respectively. The specific targets of Reptin and Pontin and the β-catenin pathway in this process remain to be determined.

Hypertrophy

Growth of the heart during embryogenesis occurs primarily through proliferation of cardiac myocytes. However, soon after birth, cardiac myocytes withdraw irreversibly from the cell cycle and subsequent growth of the heart occurs predominantly through hypertrophy rather than myocyte hyperplasia. In broad terms, there are two forms of cardiac hypertrophy, physiological, as occurs in response to exercise, and pathological, as occurs in response to abnormal stress. Stress signals that induce hypertrophy include hypertension, pressure overload, endocrine disorders, myocardial infarction, and contractile dysfunction from inherited mutations in sarcomeric or cytoskeletal proteins. Pathological hypertrophy frequently progresses to dilated cardiomyopathy (Fig. 3), which may be due, at least in part, to activation of apoptotic pathways (for review, see Kang and Izumo 2003). Certain pathological signals also cause cardiac dilatation without an intermediate hypertrophic stage. There is general agreement that cardiac hypertrophy is triggered by abnormalities in calcium homeostasis within the cardiomyocyte (for review, see Frey et al. 2000a). However, the mechanism whereby calcium, which fluctuates in concentration by orders of magnitude during each cycle of contraction and relaxation, can activate hypertrophic signaling remains vague.

Hypertrophic growth involves control at multiple molecular levels: transcription initiation, transcript elongation, and protein translation (Fig. 4). An intricate web of interconnected signaling modules has been implicated in hypertrophy of postnatal cardiomyocytes (for reviews, see Hunter and Chien 1999; Molkentin and Dorn 2001). These signaling pathways culminate in the nucleus with the posttranslational activation of a set of transcription factors, all of which have earlier roles in heart development (Fig. 4). When activated in the adult myocardium,

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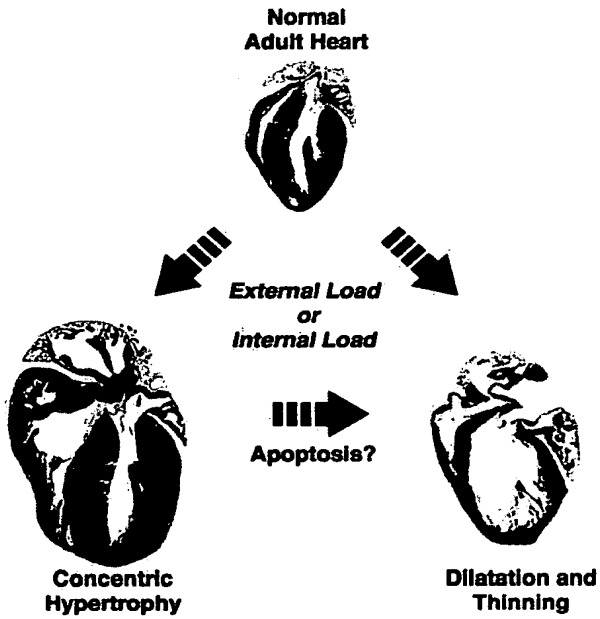

Figure 3. Growth of the adult heart by hypertrophy and dilation. The adult heart can undergo concentric hypertrophic growth that progresses to dilated cardiomyopathy or it can undergo dilatation directly through the actions of pathological signaling pathways that impose external or internal load.

these factors reawaken a "fetal" cardiac gene program. Although elements of this program might be salutary adaptations to stress initially, it has become increasingly clear, if not arguable, that the aberrant expression of fetal proteins involved in contractility, calcium handling, and myocardial energetics leads to maladaptive changes in cardiac function (Miyata et al. 2000). Several counter-regulatory mechanisms have also been identified in hypertrophic signaling circuits that provide opportunities for pharmacological and genetic interference with pathological signaling from the cytoplasm to the nucleus.

The MAP kinase module

Mitogen-activated protein (MAP) kinase signaling through each of the three terminal MAPK families (ERK, JNK, and p38) plays a key role in hypertrophy, with differing effects, respectively, on growth per se versus the fetal gene program and cardiomyocyte survival (for review, see Sugden and Clerk 1998). For instance, MAPKKK7 (also known as TGF-β activated kinase) is activated as a late response to mechanical load and is sufficient, at pathophysiological levels of function, to drive an apoptotic cardiomyopathy in mice (Zhang et al. 2000). Numerous upstream initiating triggers for this pathway have been identified, but relatively little is known of the transcriptional targets that ultimately integrate such signals. Notably, MEF2 factors are phosphorylated by MAP kinases, which augments their transcriptional activity (McKinsey et al. 2002). GATA factors and ATF transcription factors, both cofactors for SRF, also are stimulated by MAP kinase phosphorylation (Zhang et al. 2000; Liang et al. 2001; Monzen et al. 2001).

The calcineurin module

The calcium-dependent phosphatase calcineurin is an especially powerful activator of cardiac growth, both sufficient and in some cases necessary for hypertrophy in response to diverse stimuli, including the signals discussed earlier (Molkentin et al. 1998). Calcineurin, which is localized to the cytoplasm and the Z-line of the sarcomere (Frey et al. 2000b), is activated by a specific waveform of calcium. Pharmacological inhibition of calcineurin with cyclosporin A or FK-506, or genetic inhibition in calcineurin knockout mice or transgenic mice expressing dominant negative forms of the protein, abrogates cardiac growth (for review, see Leinwand 2001).

Members of the nuclear factor of activated T cells (NFATs) are among the key substrates of calcineurin (Crabtree and Olson 2002). Dephosphorylation of NFAT proteins by calcineurin results in their translocation to the nucleus where they interact with GATA and MEF2 transcription factors, providing a link between calcium signaling and fetal cardiac gene expression (Molkentin et al. 1998). It is interesting to note that NFAT proteins have also been implicated in growth of skeletal muscle in response to the cytokine interleukin 4 (IL-4; Horsley et al. 2003). Whether similar cytokine signaling pathways govern cardiac growth remains to be determined.

NFATs are rephosphorylated by GSK3, resulting in their export from the nucleus and termination of calcium-dependent transcription. Thus, GSK3 can impose a powerful restraint on prohypertrophic signaling by sustaining the phosphorylation of NFAT proteins (and probably other substrates), thereby antagonizing the actions of calcineurin. The suppression of GSK3 activity by the kinase Akt/protein kinase B provides a crossover point between the calcineurin and insulin-like growth factor (IGF)-phosphoinositide 3-kinase (PI3K) signaling pathways (Fig. 4). GSK3 has also been shown to promote nuclear export of GATA4, which would be predicted to antagonize hypertrophic signaling (Morisco et al. 2001). Consistent with this conclusion, transgenic mice that express a constitutively active form of GSK3 are resistant to intrinsic and extrinsic hypertrophic signals (Antos et al. 2002). Remarkably, despite the lack of "adaptive" growth, these mice sustain normal cardiac function in the face of cardiac stress.

Calcineurin signaling in the heart is also suppressed by a family of calcineurin regulatory proteins known as modulatory calcineurin interacting proteins (MCIPs), which interact with the calcineurin catalytic subunit to inhibit activity (Rothermel et al. 2001). MCIP1 is upregulated by calcineurin signaling through a series of NFAT sites in its promoter (Yang et al. 2000), creating a negative feedback loop to dampen calcineurin signaling. Accordingly, transgenic overexpression of MCIP in the

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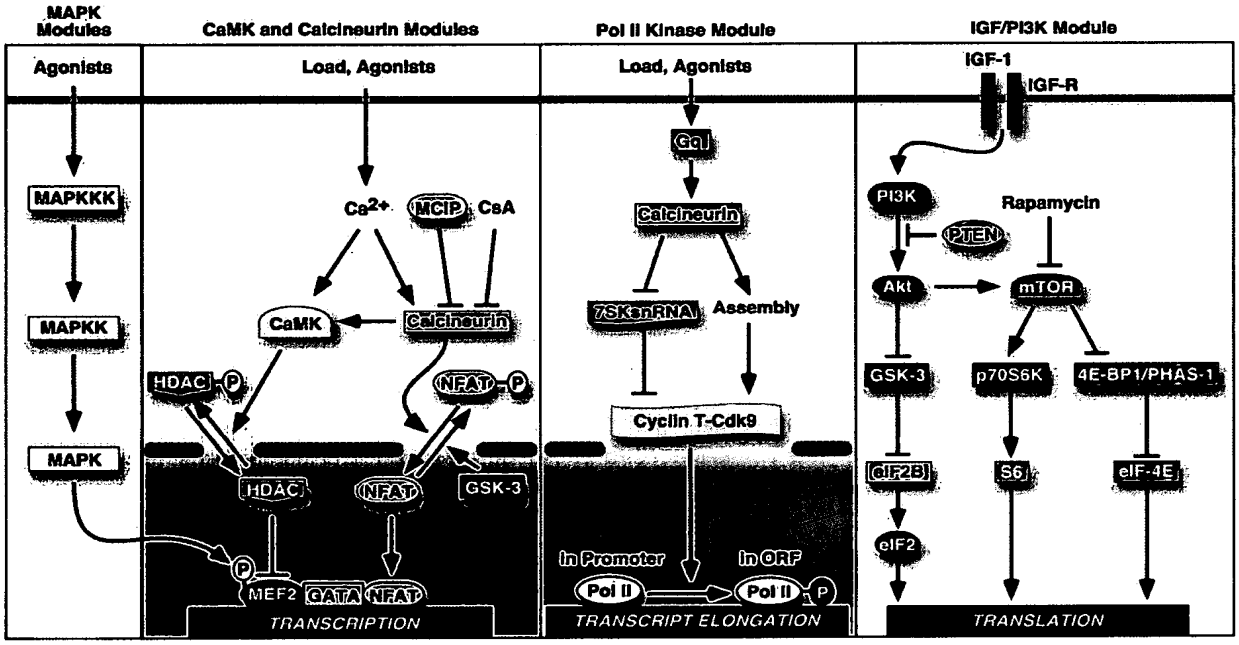

Figure 4. Hypertrophic signals leading to fetal gene activation, myocyte enlargement, or both. Four signaling modules that promote hypertrophy with activation of the fetal gene program in response to diverse agonists and load are shown. One key target of the MAPK module is MEF2, which cooperates with GATA and NFAT transcription factors to drive hypertrophy and fetal gene expression in the adult heart. The CaMK and calcineurin module uses calcineurin to dephosphorylate NFAT, which enters the nucleus to engage GATA and MEF2. CaMK signaling dephosphorylates class II HDACs, resulting in their dissociation from MEF2 and consequent potentiation of MEF2 activity. Calcineurin signaling also activates the HDAC kinase through an unknown mechanism. Calcineurin enhances transcript initiation via a cyclin T–Cdk9 complex that phosphorylates RNA polymerase II. IGF stimulates translation by the Akt kinase and its downstream effectors controlled by GSK3 and mTOR.

heart confers resistance to pressure overload, chronic adrenergic stimulation, and exercise (Rothermel et al. 2001).

These studies would predict that MCIP knockout mice might be supersensitive to hypertrophic signals and indeed they rapidly develop a lethal cardiomyopathy in response to constitutive calcineurin activation (Vega et al. 2003). However, these mice show a reduced responsiveness to pressure overload and adrenergic stimulation. These findings have led to a model in which MCIPs are both permissive for calcineurin activation, possibly by performing a chaperone function, and repressive to excessive levels of calcineurin activity. The single MCIP gene in yeast, rgs, plays a similar dual role in governing calcineurin functions (Bonilla et al. 2002).

The MEF2–HDAC module

The MEF2 transcription factor also serves as a target for hypertrophic signaling (Lu et al. 2000; Nadruz et al. 2003). In the normal adult myocardium, MEF2 exhibits only basal activity, which is likely to be required for maintenance of contractile protein gene expression and energy metabolism (Passier et al. 2000; Naya et al. 2002). Stress signaling stimulates the transcriptional activity of MEF2 by causing the nuclear export of class II histone deacetylases (HDACs), which associate with MEF2 and suppress its activity (Fig. 4; McKinsey et al. 2000). Nuclear export of class II HDACs is mediated by a stress-responsive protein kinase with the same substrate specificity of CaM kinase, a potent activator of MEF2 and hypertrophy (Zhang et al. 2002). These kinases phosphorylate two conserved sites in HDACs that create docking sites for the 14-3-3 family of chaperone proteins. Binding of 14-3-3 to these phosphoacceptors results in dissociation from MEF2 and nuclear export with consequent derepression of MEF2 activity (for review, see McKinsey et al. 2002).

Consistent with the notion that class II HDACs act as suppressors of cardiac growth and fetal gene activation, mice lacking HDAC9 are supersensitive to stress signals (Zhang et al. 2002). Conversely, expression of signal-resistant HDAC mutants in primary cardiomyocytes is sufficient to suppress fetal gene activation in response to hypertrophic signaling. Paradoxically, HDAC inhibitors currently being tested in clinical trials as anticancer agents block cardiomyocyte hypertrophy and fetal gene expression (Antos et al. 2003). The basis for this anticar-

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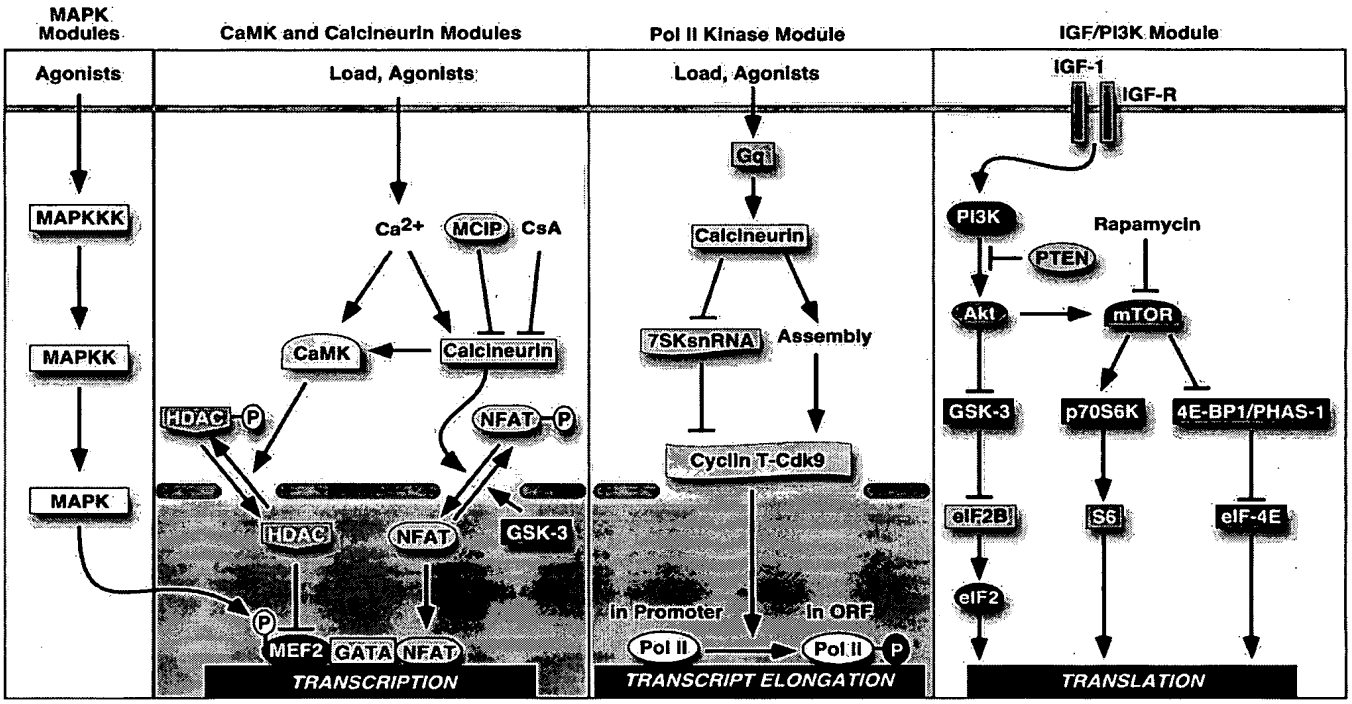

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diac growth effect remains to be defined. However, it is interesting to speculate that this activity reflects a pro-cardiac growth function of certain HDACs that are dominant over the growth inhibitory function of HDAC9 and other class II HDACs.

The cyclin T–cdk9 pathway

An additional mechanism to control protein accumulation in hypertrophic cardiomyocytes involves the global increase in RNA transcription via hyperphosphorylation of the C-terminal domain (CTD) of RNA polymerase II (Fig. 4; Sano et al. 2002; Sano and Schneider 2003). Whereas unphosphorylated polII is the form recruited to promoters for transcript initiation, phosphorylation of polII is implicated in productive transcript elongation, that is, the ability of polII to escape the promoter-proximal region and enter the open reading frame (Orphanides and Reinberg 2002). Diverse hypertrophic signals including the agonist endothelin-1, mechanical load, the G protein Gαq, and calcineurin have been shown to stimulate activity of cyclin-dependent protein kinase 9 (cdk9), which phosphorylates the polII CTD, by inducing the dissociation of its endogenous inhibitor, a small nuclear RNA inhibitor (7SK; Sano et al. 2002). Blockade of 7SK expression with antisense oligonucleotides resulted in spontaneous hypertrophy, as did transgenic overexpression of the cdk9 activator cyclin T. Chronic hypertrophic signals activate Cdk9 through an additional mechanism, as well, involving increased assembly of the cyclin T–Cdk9 complex (M. Sano and M. Schneider, unpubl.).

The IGF–PI3K module

Growth of the heart after birth has been shown to involve the IGF signaling pathway, which signals through PI3K to the Akt/protein kinase B (Shioi et al. 2002). This same pathway controls cell, organ, and body size in *Drosophila* (Cantley 2002). Manipulation of this pathway by overexpression of IGF1 induces cardiac hypertrophy (Delaughter et al. 1999), although cardiomyocyte hyperplasia has also been reported in some studies (Reiss et al. 1996). A key downstream target of Akt in this pathway is GSK3 (Fig. 4). Phosphorylation by Akt inactivates GSK3 and results in stimulation of protein synthesis. In addition, Akt has been proposed to activate the mammalian target of rapamycin (mTOR), which stimulates protein synthesis by phosphorylating two key effectors, p70 S6 kinase and the elongation factor 4E (eIF4E) inhibitor PHAS-1/4E-BP1 (for reviews, see Schmelzle and Hall 2000; Gingras et al. 2001). Consistent with the central role of this pathway in cardiac growth, hypertrophy in response to pressure overload and various agonists can be attenuated by rapamycin, a lipophilic macrolide that inhibits mTOR activity (Sadoshima and Izumo 1995; Boluyt et al. 1997; Shioi et al. 2003). The lipid phosphatase PTEN also suppresses this pathway by antagonizing the action of PI3K. Accordingly, cardiac-specific deletion of PTEN in mice results in cardiac hypertrophy (Crackower et al. 2002). This pathway has also been implicated in skeletal muscle hypertrophy, suggesting common mechanisms for growth of striated muscles postnatally (Bodine et al. 2001).

Regeneration

Although hypertrophy can increase cardiac mass in response to stress, it is inadequate to restore cardiac function following myocyte loss, as occurs following myocardial infarction and ischemia-reperfusion injury. In contrast to mammalian skeletal muscle, which can regenerate following injury, the mammalian heart has limited restorative potential. Classically, regeneration of skeletal muscle is achieved through activation of a population of quiescent myogenic precursors known as "satellite" cells associated with the myofiber basal lamina; a second mechanism for reconstitution of skeletal muscle involves multipotent adult stem cells, discovered only recently (for review, see Seale et al. 2001). Satellite cells are activated in response to injury and are recruited into the preexisting myofiber where they support myofiber repair. The heart has not been thought to contain an equivalent satellite cell population. The inability of injured myocardium to repair itself more fully does not appear to reflect a fundamental property of cardiac tissue because the hearts of newts and zebrafish are able to regenerate following injury. Because fetal cardiac myocytes contain functional sarcomeres and are highly proliferative, the mere existence of a contractile apparatus per se cannot account for the inability of adult cardiac myocytes to undergo cytokinesis. However, the sarcomeres of adult cardiomyocytes are more highly ordered and stable than those of fetal myocytes, as evidenced by the lack of M-lines in fetal myocytes. Similarly, cardiomyocytes in newts and zebrafish are more analogous to those of mammalian embryos with respect to their sarcomere organization.

The mechanisms responsible for the blockade to proliferation in terminally differentiated cardiomyocytes are unknown. However, numerous studies have implicated tumor pocket proteins, such as Rb, and cyclin-dependent kinase inhibitors in this process (for reviews, see MacLellan and Schneider 2000; Pasumarthi and Field 2002). Because knockout mice lacking various components of these cell cycle checkpoints show only modest increases in myocyte cell numbers (Poolman et al. 1999), there are likely to be redundant mechanisms involved in preventing adult myocytes from reentering the cell cycle. This would also explain why cardiac tumors, referred to as myxomas, are extraordinarily rare.

Genetic models with altered capacity for cardiac repair

Notably, several mouse mutations, spontaneous and engineered, have altered capacity for cardiac cell regeneration and myocardial repair. An especially instructive one affects the activity of telomerase, which is associated

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with the potential for long-term self-renewal in "immortal" adult cells—stem cells, tumor cells, and germ cells (Blackburn 2001; Oh et al. 2001). Telomeres enable cells to avoid the end-replication problem (the inability of DNA polymerase to completely replicate DNA ends), using a specific RNA template [telomerase RNA component (TERC)] and an RNA-dependent DNA polymerase, telomerase reverse transcriptase (TERT). Deletion of the RNA template, TERC, in mice abolishes telomerase activity and causes progressive telomere shortening by 3–5 kb pairs per generation (Blasco et al. 1997). After three to six generations, this null genotype results in critical telomere dysfunction, evidenced by impaired longevity and defects, especially in highly proliferative organs (Rudolph et al. 1999). Cardiac myocytes from late-generation $TERC^{-/-}$ mice showed significant telomere shortening and impaired ventricular pump function, ventricular thinning, and apoptosis (Leri et al. 2003).

Conversely, in urodele amphibians such as the newt, cardiac repair—like limb and lens regeneration—is strikingly robust (Oberpriller et al. 1995; Brockes and Kumar 2002). The MRL mouse strain exhibits the remarkably similar feature of cell replacement without scarring, after cardiac injury (Leferovich et al. 2001). First encountered as the perfect and hereditable healing of ear hole punch wounds, the regenerative phenotype in this line appears to involve the collective action of 20 or more genetic loci (McBrearty et al. 1998; Blankenhorn et al. 2003), and is possibly mediated by differences in the activity of matrix metalloproteinases and their inhibitors (Gourevitch et al. 2003). The resemblance of regeneration in this mouse model to epimorphic limb regrowth in newts (Brockes and Kumar 2002) has suggested a mechanism akin to the regeneration blastema: local dedifferentiation of cells underlying the wound, followed by growth and then reversal to the differentiated cell type (Leferovich et al. 2001; Gourevitch et al. 2003). It has been suggested recently that thrombin protease activity after tissue injury might be a pivotal determinant for the onset of regenerative growth (Imokawa and Brockes 2003).

Despite the precedent of heart regeneration in amphibians (Oberpriller et al. 1995), none of the urodeles is tractable genetically. In contrast, recent studies with the zebrafish provide a potential genetic model for dissecting this problem (Poss et al. 2002a). Amputation of the apical ventricle led to rapid clotting at the site, ingrowth of cardiac myofibers over the following weeks, robust myocyte proliferation, and complete replacement of the clot within 60 d, with little or no fibrosis. In a temperature-sensitive mutant of a mitotic checkpoint kinase, mps1, cardiac restitution was incomplete and a reciprocal increase in fibrosis was observed, suggesting that myocyte proliferation determines the extent of scarring (Poss et al. 2002b). Such regeneration, although dramatic, is clearly distinct from the form of regeneration involved in complete reconstitution of a limb, for example, following amputation. It is unknown whether a cell-autonomous effect in cardiomyocytes themselves or in an as-yet-unknown progenitor cell is the primary target to explain this phenotype. Alternatively, an extract from regenerating newt limbs drives mouse myotubes to dedifferentiate and reenter the cell cycle (McGann et al. 2001), and deficiencies in cardiac repair could arise from defects in such pathways.

Strategies for cardiac repair

Cell replacement

One potential approach for cardiac repair that bypasses cell cycle constraints is to implant additional myocytes into the injured heart. Work with fetal cardiac myocytes established the overall feasibility of this approach, including formation of at least nascent intercalated discs, as required for appropriate connectivity (Soonpaa et al. 1994; Koh et al. 1995; Reinecke et al. 1999). Fetal cardiac myocytes appear to survive better after grafting than do adult ones (Reinecke et al. 1999), prevent the adverse dilatation of ventricular muscle when implanted after ischemic damage, and improve ventricular pump function. However, the actual basis for remediating organ-level function became surprisingly ambiguous, on the discovery that various noncontractile cells were likewise beneficial in enhancing the function of surviving cardiomyocytes (Kocher et al. 2001; Kawamoto et al. 2003), stabilizing or diminishing the myocardial scar, and preventing late myocardial thinning and dilatation. Few side-by-side comparisons, though, have tested directly the premise that contractile cells are better for the heart than noncontractile ones. It has been suggested that ventricular relaxation (diastole) can be improved almost regardless of cell type, whereas ventricular contraction (systole) more specifically requires contractile cells (Sakai et al. 1999; Hutcheson et al. 2000); notably, though, systolic function was improved by marrow-derived angioblasts (Kocher et al. 2001).

Conceivably, skeletal myocytes might substitute for cardiac ones (Taylor et al. 1998), and clinical trials with human skeletal myoblasts have been initiated (Hagege et al. 2003) despite the cells' inherent differences from ventricular myocytes in ion transport, connectivity, and contractile proteins, and despite lack of evidence for their transdifferentiation into cardiac muscle following engraftment (Reinecke et al. 2002). Indeed, as might be anticipated, cardiac arrhythmias have already emerged as a possible threat to this strategy (Menasché et al. 2003). Hence, cell transplantation is likely to be more efficacious with cells that are (or can become) cardiac myocytes themselves.

Luring cells to a cardiac fate

An alternative approach for cardiac repair is to convert noncardiac cells to a cardiac cell fate. In principle, this could occur through the transdifferentiation of cardiac fibroblasts, which comprise most of the cells of the myocardium, or through the generation of cardiac myocytes from multipotential stem cells in vitro, followed by cel-

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lular transplantation. Mouse ES cells can give rise efficiently to cardiac myocytes in culture (Doetschman et al. 1985; Robbins et al. 1990). This property has been useful for testing of candidate factors that might regulate entry into a cardiogenic pathway: leukemia inhibitory factor as a repressor (Bader et al. 2001), RA as an inducer (Wobus et al. 1997). The use of cardiac-restricted promoters to express selectable markers has also enabled the isolation of purified populations of cardiac muscle cells from this infinitely renewable source (Klug et al. 1996). The feasibility of generating rhythmically beating cardiomyocytes from human ES cells has also been substantiated (Kehat et al. 2001).

Less expected was the evidence now accumulating that adult, somatic cells might be recruited to the cardiomyocyte lineage (Fig. 5). The largest body of work deals with bone marrow-derived cells of several kinds. Despite their very low prevalence (500 per million total bone marrow cells), the so-called "side population" (SP) of $CD34^-$ $c\text{-}Kit^+$ cells, designated on the basis of Hoechst dye efflux, account for the majority of long-term self-renewing hematopoietic stem cells, and can reconstitute all of the hematopoietic lineages in mice (Goodell et al. 1996). Transplanting adult bone marrow SP cells marked by lacZ expression into lethally irradiated recipients demonstrated the recruitment of lacZ-positive marrow-derived cells into vascular endothelium and cardiomyocytes, after coronary artery occlusion (Jackson et al. 2001). Homing and stable engraftment under these conditions occurred specifically at the border between infarcted and normal myocardium, and was never seen in the absence of injury. Although the molecular cues that guide homing from the marrow compartment remain to be proven, it is worth noting that the infarct "border zone" preferentially expresses numerous chemotactic signals, including cytokines and adhesion molecules (Dewald et al. 2003). From a purely developmental perspective, it is intriguing that marrow-derived SP cells, although lacking markers of differentiated endothelium (factor VIII, VE-cadherin, factor VIII) and of endothelial progenitor cells (vascular-endothelial growth factor receptors), express high levels of angiopoietin-1, its receptor Tie-2, VEGF-A, and platelet-endothelial cell adhesion molecule-1 (PECAM-1, CD31; Jackson et al. 2001). Many of these features are reminiscent of the hemangioblast, a progenitor shared by hematopoietic and endothelial cells during embryogenesis (Minasi et al. 2002).

From a therapeutic perspective, the rate for engraftment directly from bone marrow into myocardium (0.02% of all cardiomyocytes) was exciting as proof of a

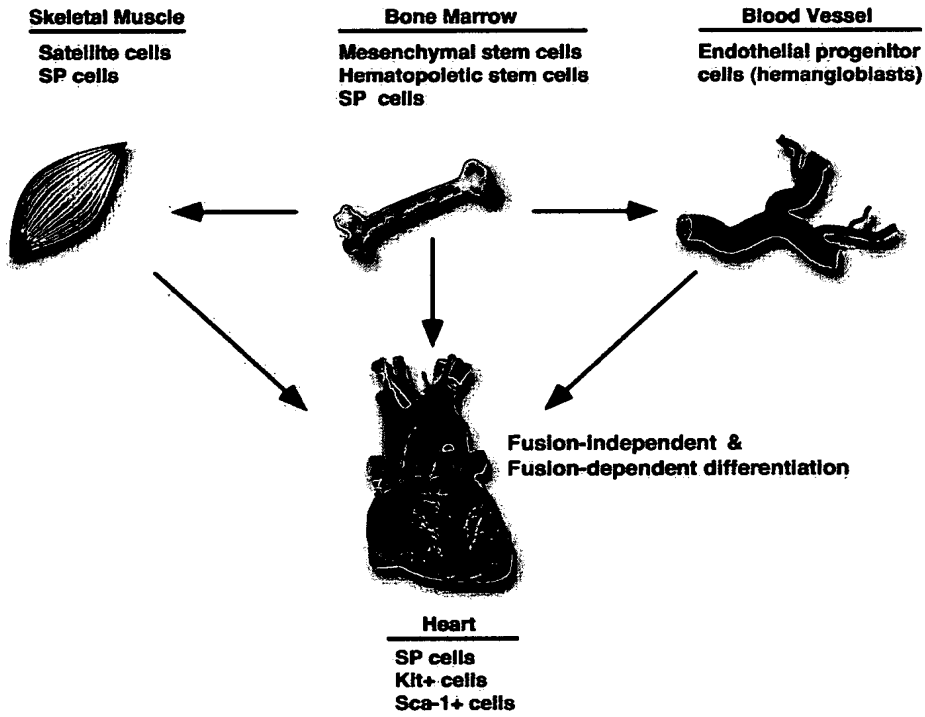

Figure 5. Stem cells intrinsic to different tissues and their transport between tissues. Intrinsic progenitor cells are shown for each of the four tissues. Arrows to the heart denote human clinical trials, now underway, using skeletal myoblasts (Menasché et al. 2003), bone marrow cells (Strauer et al. 2002; Stamm et al. 2003), and endothelial progenitor cells (Assmus et al. 2002) for cardiac repair. Arrows from bone marrow denote its role as a source of skeletal muscle SP cells (McKinney-Freeman et al. 2002), endothelial progenitor cells (Takahashi et al. 1999), and cardiomyocytes (Jackson et al. 2001).

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novel biology, but unlikely to comprise effective regeneration on its own (Jackson et al. 2001). Hence, augmenting the marrow-derived stem cells in number or state of activation could be logical. In one such study, c-Kit$^+$ bone marrow cells were injected directly into the ventricular wall of mice adjacent to the infarct, resulting in migration into the damaged region, differentiation into cardiomyocytes and vascular cells, and partial replacement of the necrotic myocardium (Orlic et al. 2001a). The complementary strategy of mobilizing endogenous marrow-derived cells was also reportedly effective, using stem cell factor plus granulocyte-colony stimulating factor (Orlic et al. 2001b). The therapeutic appeal of this approach would be limited if removal of the spleen were necessary clinically, as was done for these experiments. Nevertheless, this finding points to the potential to mobilize and recruit circulating progenitors to the injured heart.

Adult bone marrow also contains mesenchymal stem cells, which differentiate into many mesenchymal tissue types, such as bone, marrow stroma, cartilage, tendons, ligaments, fat, and skeletal muscle. These marrow-derived stromal cells, lacking hematopoietic markers like CD34 and CD45, were found to contain high levels of telomerase and to have multilineage potential even as clonal cells (Pittenger et al. 1999). When injected into the left ventricular cavity of immunodeficient SCID mice, human mesenchymal stem cells were found chiefly in the liver, spleen, and lungs, but low levels of engraftment were seen in myocardium, with expression of many cardiac contractile proteins, cross-striations indicative of sarcomere organization, and the cardiac-specific regulator of calcium uptake into sarcoplasmic reticulum (Toma et al. 2002). More prevalent engraftment by these cells occurred when directly injected into the injured ventricular wall in pigs, after acute ischemic damage (Shake et al. 2002).

Endothelial cells, or at least their less differentiated progenitors, offer a different source for adult cardiomyocytes. Endothelial cells from the mouse dorsal aorta at E9 were grown as clonal isolates, fluorescently labeled with replication-defective lentivirus, and cocultured with neonatal rat cardiomyocytes (Condorelli et al. 2001). After 5 d, 10% of the fluorescent cells were positive for sarcomeric myosin heavy chains and cardiac troponin-I. Similar conclusions were reached using human umbilical vein endothelial cells, including the transient coexpression of sarcomeric myosin and von Willebrand factor (Condorelli et al. 2001). Interestingly, most of the double-labeled cells were contiguous with cardiomyocytes; cardiomyocyte-conditioned medium did not induce conversion, suggesting that cell–cell contact might be needed.

Analogously, adult human endothelial progenitor cells, derived from peripheral blood mononuclear cells, also were reported to convert into cardiomyocytes on coculture with rat cardiomyocytes, as did CD34$^+$ hematopoietic progenitor cells (Badorff et al. 2003). Because even killed cardiomyocytes were sufficient for this instructive signal and because conditioned medium was not, the best inference is a requirement for cell–cell contact or, alternatively, a secreted signal that remains associated with the extracellular matrix.

Based on these summarized experimental data, multiple clinical trials have been initiated using bone marrow-derived cells or endothelial progenitor cells for cardiac repair. Initial experience has been reported already for several of these (Assmus et al. 2002; Strauer et al. 2002; Hagege et al. 2003; Stamm et al. 2003; Tse et al. 2003), although the emphasis at this early stage has been necessarily to prove safety, not effectiveness.

Driving cardiac myogenesis in therapeutic settings

Currently, much more is known about which cells have cardiogenic potential for cardiac cell grafting than about what actual cues or pathways guide the cells' specification (or respecification, if already differentiated). It is, for example, unclear whether the same signals that specify cardiac cell fate in the early embryo (e.g., BMP's, anti-Wnts or Wnts) would also suffice in the adult context of cardiac "neogenesis". In a noteworthy early example of what the future can be hoped to bring (Behfar et al. 2002), paracrine pathways have been dissected for mouse ES cell conversion to the cardiac phenotype. In culture, cardiogenesis was responsive to exogenous TGF-β or BMP2, promoted by coculture of the ES cells with cardiomyocytes and abolished by latency-associated peptide and noggin, inhibitors of TGF-β and BMPs, respectively. Although alternative targets for the inhibitors should be considered, this implicates TGF-β and BMPs as cardiogenic paracrine signals from differentiated myocytes. This conclusion was next tested in vivo, after cardiac grafting with ES cells bearing a heart-specific cyan fluorescent protein gene: cardiogenic differentiation in situ was prevented by both noggin and by a dominant-negative TGF-β receptor (Behfar et al. 2002).

The notion of cellular plasticity is subject to several important caveats, reviewed in recent publications (Anderson et al. 2001; Blau et al. 2001; Seale et al. 2001; Verfaillie 2002).

Cardiac progenitor cells from adult hearts

Although cardiac muscle has not been thought to harbor a tissue-resident progenitor cell population akin to skeletal muscle satellite cells (Seale et al. 2001), recent studies have suggested the existence of a population of "immature" cardiac myocytes that may be activated in response to injury and recruited for tissue repair. One route to the discovery of candidate cells and molecules for this role in myocardium has been efflux of Hoechst dye 33342, as used to denote SP cells in bone marrow (Asakura and Rudnicki 2002; Hierlihy et al. 2002), or expression of the responsible ATP-binding cassette transport proteins (Martin et al. 2003). A second is the use of stem cell surface markers: for instance, a c-kit$^+$ population from adult heart migrates into the central area of necrosis, expresses cardiac differentiation mark-

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ers, and improves mechanical function when injected into injured myocardium (Bartucchi et al. 2002). Alternatively, it may be possible to mobilize such cells to damaged myocardium from their niche within the healthy heart, using suitable cytokines or chemokines.

Telomerase has provided a third means of identifying progenitor cells within adult myocardium (Oh et al. 2003). Down-regulation of TERT occurs in the myocardium after birth, whereas forced expression of TERT is sufficient to prolong cardiac myocyte cycling (Oh et al. 2001) and confer protection from stress-induced telomere erosion and apoptosis (Oh et al. 2003). The small but measurable residual telomerase activity in normal adult hearts prompts the question of whether a telomerase-positive subpopulation exists. If so, do such cells have cardiogenic potential, and what mechanisms might be involved in their recruitment to the cardiac differentiation pathway?

Telomerase activity in adult hearts was found to be localized to small interstitial cells expressing stem cell antigen-1 (Sca-1), which lacked the hallmarks of either hematopoietic stem cells (c-kit, CD45, CD34) or endothelial progenitor cells (CD45, CD34, Flk-1, Flt-1; Oh et al. 2003). Although purified cardiac Sca-1$^+$ cells fail to express cardiac structural proteins, they express many cardiogenic transcription factors. Using a Cre/Lox donor/recipient pair, cardiac Sca-1$^+$ cells were shown to home specifically to infarcted myocardium and to differentiate in situ. Half the donor-derived cardiomyocytes arose autonomously of fusion with endogenous myocytes, but half were chimeric, indicating fusion had occurred. Such findings reinforce the importance of analyzing both modes of differentiation after grafting, as has been seen in other systems (Medvinsky and Smith 2003). The molecular circuitry for cardiogenesis has begun to be dissected in this system, which might both overlap or differ from normal development in its details. However, as in virtually all developmental systems, at least a partial requirement for BMPs exists.

Regulating the cardiac cell cycle

From an applied perspective, how might fundamental knowledge of heart muscle cells' development be exploited to enhance progenitor cells' number, stable engraftment, differentiation, or function? One appealing and logical approach is to delay terminal differentiation and promote cardiomyocyte cycling, controllably. Tumor suppressor "pocket proteins," of which the retinoblastoma gene product is the founding and best-studied member, are thought to mediate terminal differentiation and growth arrest (Novitch et al. 1999; MacLellan and Schneider 2000; Pasumarthi and Field 2002). Recently, it has been possible to create cardiomyocyte lines from ventricular myocardium, by conditional (Cre-deletable) expression of SV40 large T antigen under the transcriptional control of cardiac-specific elements from the *Nkx-2.5* gene (Rybkin et al. 2003); deletion of the transforming gene resulted in growth arrest and susceptibility to differentiating signals including BMPs. Immortalization by SV40 large T antigen but not a related protein, adenoviral E1A, likely depends on the ability of large T antigen to interfere not just with pocket proteins, but also the tumor suppressor p53 and a BH3-only apoptotic protein, p193 (Pasumarthi and Field 2002).

Looking to the future

In summary, the size of the heart is governed by three general mechanisms: developmental growth, hypertrophy, and regeneration. Each of these mechanisms and their associated regulatory circuits are used to varying degrees during different stages of cardiac growth, development, and disease. Despite the ability of the heart to modulate its size by these mechanisms, all of the existing endogenous mechanisms for repair, acting collectively, are obviously inadequate—when unassisted—to restore function fully to the adult heart following injury. We envision that more complete restoration of function to the injured heart, and perhaps the maintenance of cardiac function despite aging, will benefit from the exploitation of developmental control mechanisms. One set of therapeutic targets will involve strategies for increasing cardiac muscle cell number: coaxing noncardiac cells to adopt a cardiac fate, selectively amplifying the small subpopulation of immature cardiomyocytes that resides in the adult heart, or overcoming the barrier to proliferation in terminally differentiated cardiomyocytes. Lest these seem far-fetched and remote, it needs to be recalled that clinical trials implanting cells of several kinds have been implemented.

A second approach will involve strategies that suppress adverse adaptations and, perhaps, enhance the salutary ones. Indeed, molecular dissection of the intracellular circuits that couple stress signals to developmental transcription factors in the hypertrophied and failing heart have revealed nodal points currently being exploited as targets for drug discovery. Thus, a mechanistic understanding of how the heart sizes itself up during development and disease promises to yield unanticipated therapeutic targets and novel strategies for accomplishing the ambitious goals of cardiac repair and regeneration, ensuring that the beat goes on.

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